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Characterization of the Peptide Binding Motif of a Rhesus MHC Class I Molecule (Mamu-A*01) That Binds an Immunodominant CTL Epitope from Simian Immunodeficiency Virus¹

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The majority of immunogenic CTL epitopes bind to MHC class I molecules with high affinity. However, peptides longer or shorter than the optimal epitope rarely bind with high affinity. Therefore, identification of optimal CTL epitopes from pathogens ultimately be critical for inducing strong CTL responses and developing epitope-based vaccines. The SIV-infected rhesus macaque is an excellent animal model for HIV infection of humans. Although a number of CTL epitopes have been mapped in SIV-infected rhesus macaques, the optimal epitopes have not been well defined, and their anchor residues are unknown. We have now defined the optimal SIV gag CTL epitope restricted by the rhesus MHC class I molecule Mamu-A*01 and defined a general peptide binding motif for this molecule that is characterized by a dominant position 3 anchor (proline). We used peptide elution sequencing, peptide binding assays, and bulk and clonal CTL assays to demonstrate that the optimal Mamu-A*01-restricted gag CTL epitope was CTPYDINQM₁₈₁₋₁₈₉. Mamu-A*01 is unique in that it is found at a high frequency in rhesus macaques, all SIV-infected Mamu-A*01-positive rhesus macaques studied to date develop an immunodominant gag-specific CTL response restricted by this molecule. Identification of the optimal SIV gag CTL epitope will be critical for a variety of studies designed to induce CD8⁺ CTL responses specific for SIV in the rhesus macaque. *The Journal of Immunology*, 1998, 160: 6062-6071.

Progress toward the development of a vaccine for HIV has been hindered by the lack of well-defined animal models with which to study HIV infection of humans. SIV infection of the rhesus macaque represents perhaps the best animal model for HIV infection of humans (1, 2). The nucleotide sequences of the SIVs are closely related to those of HIV-1 and -2 (3, 4), and SIV and HIV have similar tropisms for CD4⁺ T cells (5, 6). Importantly, infection with SIV causes an AIDS-like disease in the majority of infected macaques by 1 yr postinoculation (7), making SIV infection of macaques the most cost-effective animal model to test vaccine efficacy in vivo.

Because of the similarity of the immune systems of macaques and humans, SIV infection of macaques is also an excellent model to study the immunology of HIV infection of humans. Many macaque genes that encode proteins important in the functioning of the immune system are remarkably similar to their human homo-

logues. Specifically, homologues of the human MHC class I (8) and class II (10), and TCR genes (11, 12) are all found in the macaque. Interestingly, macaque and human MHC class I molecules bind peptides derived from similar regions of the gag and env proteins of HIV and SIV (13-19). Similarities in peptide binding ability of MHC proteins are also evident at the class II loci, with rhesus macaque lymphocytes able to present a mycobacterially derived peptide to a human T cell clone (20). Taken together, these data suggest that some elements of Ag processing as well as the peptide binding specificities of MHC molecules may have been conserved between humans and macaques.

The cell-mediated immune response may play an important role in the containment of HIV in infected individuals, especially during the first few weeks postinfection (21-23). Recent evidence from studies of HIV-infected patients also implicates the role of CTL in containing the virus later in infection (24-26). Evidence for transient CTL activity in several infants born to HIV-infected mothers suggests that CTLs may also be important in protection against infection with HIV (27, 28). More recently, it has been shown that mutation of an immunodominant HLA-B*27-restricted gag CTL epitope, after 9 to 12 yr of stability in two HLA-B*27-positive HIV-infected individuals, coincided with an increase in viral load, a decline in CD4⁺ T cells, and progression to AIDS (29). Rapid escape from CTL recognition has also recently been demonstrated in an individual that made a strong response to an immunodominant gp160 peptide (30). The rapid evolution of recognized variants in this and other HIV-infected individuals (31, 32) provides strong evidence for the ability of CTL to suppress viral replication in vivo.

Several studies in humans, chimpanzees, and macaques have suggested that strong humoral and cellular immune responses

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HIV and SIV can be generated through several different vaccination strategies (33–39). Indeed, DNA-encoded subunit vaccines have protected chimpanzees from high dose HIV-1 challenge (40). Infection of rhesus macaques with attenuated *nef* deletion mutants or SIV or previous exposure to HIV-2 has also been shown to protect these animals from subsequent challenges with pathogenic viruses (35, 41, 42). Furthermore, vaccination of cynomolgus macaques with vaccinia-expressing *nef* elicited high levels of CTLs in several animals, with one animal being protected against SIV challenge (43). These studies suggest that it may be possible to induce a protective immune response and provide the rationale to further explore whether CTLs can protect against AIDS virus infection in an animal model.

The majority of vaccine strategies designed against HIV and SIV induce both cellular and humoral immune responses. As such, it has been difficult to distinguish between the role of antiviral CTLs and virus-specific Abs in protection from infection or in controlling viral loads. To address this issue it will be important to stimulate CTLs in the absence of an Ab response through vaccination with those minimal regions of the virus that encode CTL epitopes. The rhesus macaque MHC class I molecule Mamu (*Macaca mulatta*)-A*01 presents a peptide from the SIV gag protein (16). This epitope has been used as a 12 mer in vaccination strategies and for stimulating CTLs to viral variants from SIV-infected animals (44, 45). However, the optimal epitope, defined as the peptide that binds with the highest affinity or that is able to sensitize target cells for lysis at low concentrations, has not been identified. In most cases, immunogenic peptides seem to be high affinity binders, and peptides that are shorter or longer than the optimal epitope generally bind with lower affinity (46, 47). Vaccination with the optimal CTL epitope, therefore, may be more efficient at inducing a strong CTL response than vaccination with longer peptides. In this study we have identified the peptide binding motif of Mamu-A*01 using peptide elution. Additionally, we have defined the optimal Mamu-A*01-restricted CTL epitope contained in the SIV gag protein using sets of overlapping peptides in live cell binding assays and in CTL assays. Identification of the SIV gag-derived CTL epitope, bound and presented by Mamu-A*01, will be important to the development of vaccine strategies designed to induce CD8⁺ CTL responses against SIV in the rhesus macaque.

Materials and Methods

Molecular cloning of MHC class I cDNAs

LP XhoI (5'-GCC TCG AGA TGS CSG TCA YGG CKC CCC GAA SYS TC-3') and 3'H3 (GCA AGC TTA GTC CCA CAC AAG GCA GCT GC-3') primers were used to amplify Mamu-A*01 using the PCR from a previously described plasmid containing the rhesus MHC class I cDNA (16). After amplification, the PCR product was ligated into pSP72 (Promega, Madison, WI), which was used to transform bacterial SCS1 cells. Plasmid minipreps were then sequenced using an Applied Biosystems 373 automated sequencer (Applied Biosystems, Foster City, CA) to obtain full-length sequence. A minimum of three copies of numerous cDNAs were sequenced and analyzed with the use of software from FBI (New Haven, CT) and Applied Biosystems. A clone containing the consensus sequence for Mamu-A*01 was selected for transfection, and the cDNA was then subcloned into the pKG5 expression vector (gift from Andrew McMichael, Oxford University, Oxford, U.K.).

Stable transfection of Mamu-A*01 into the 721.221 cell line

The pKG5 vector encoding Mamu-A*01 was electroporated into the 721.221 cell line, a cloned EBV-transformed B LCL with homozygous deletions of the MHC class I loci (48). 721.221 cells (5×10^6) were transfected in a 1-cm electroporation cuvette with 10 μ g of plasmid DNA.

Electroporation was conducted with a Zapper (Medical Electronics Shop, University of Wisconsin, Madison, WI) at 1250 V and a capacitance of 450 μ F. The cells were then incubated for 2 days at 37°C in RPMI 1640 culture medium supplemented with penicillin (50 U/ml), streptomycin (50 μ g/ml), L-glutamine (2 mM), 5% defined FBS (HyClone, Logan, UT), and 10% defined/supplemented bovine calf serum (HyClone). On day 3 the cells were placed under selection by feeding with culture medium containing 1.5 mg/ml G418 (Life Technologies, Grand Island, NY). Approximately 4 wk later, viable transfectants were tested for HLA surface expression by flow cytometry using the W6/32 mAb directly conjugated to FITC (Sigma, St. Louis, MO). The transfectant with the highest level of MHC class I expression was selected to be grown for peptide elution studies.

Affinity purification of Mamu-A*01

MHC class I molecules were purified from the surface of Mamu-A*01-transfected 721.221 cells according to a modified protocol (49). Briefly, 4×10^9 transfected 721.221 cells were washed in cold HBSS (Life Technologies), harvested, and then frozen until needed. Thawed cells were then resuspended in 100 ml of 1% Nonidet P-40 lysis buffer containing 0.25% sodium deoxycholate, 174 μ g/ml PMSF, 5 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A, 20 μ g/ml iodoacetamide, 0.2% sodium azide, and 0.003 μ g/ml EDTA. Cell lysates were incubated at 4°C for 1 h, centrifuged at $100,000 \times g$ at 4°C to remove cellular debris, and then filtered sequentially through 0.8- and 0.22- μ m pore size Nalgene filters (Nalge, Rochester, NY) to remove any remaining lipids. Filtered lysates were then passed twice over an LB3.1 (anti-class I Ab)-coupled protein A-Sepharose column to preclear the lysate. The flowthrough was passed twice over two consecutive W6/32-coupled columns (mAb W6/32, a gift from D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA) to specifically bind MHC class I molecules. The protein A beads of the W6/32 columns were then washed separately: twice with lysis buffer (without protease inhibitors), twice with a high salt buffer (1 M NaCl and 20 mM Tris, pH 8.0), and twice with a no salt buffer (20 mM Tris, pH 8.0).

Purification of MHC class I bound peptides

The MHC heavy chain β_2m /peptide complexes were eluted from the protein A beads by incubation in 0.2 N acetic acid (49). The beads were then briefly centrifuged, the supernatant transferred to a new tube, and the process was repeated. One hundred microliters of glacial acetic acid was then added to each tube to allow for dissociation of the MHC heavy chain/ β_2m /peptide complexes. MHC class I heavy chains, β_2m , and W6/32 Abs were then separated from the peptides by centrifugation through an Ultrafree-CL filter (5000 NMWL, Millipore, Bedford, MA). Peptide yields were determined by quantitation of Mamu-A*01 heavy chain using SDS-PAGE.

HPLC fractionation and automated Edman degradation sequencing of peptides

The peptide-containing eluate that passed through the 5000 m.w. filter was dried down to 100 μ l. Acid-eluted peptides were separated by HPLC using a reverse-phase Beckman Ultrasphere C₁₈ 4.6-mm \times 250-mm column and a trifluoroacetic acid (TFA)/acetonitrile water gradient consisting of a mixture of two mobile phases. Mobile phase A consisted of 5% acetonitrile in water with 0.1% TFA, while phase B consisted of 50% acetonitrile in water with 0.1% TFA. The gradient varied from 100% mobile phase A to 100% phase B over 70 min at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals, and peptides eluting between fractions 15 and 55 were collected, pooled, and subjected to Edman degradation sequencing on a high sensitivity Procise cLC sequencer (W. M. Keck Foundation, Biotechnology Resource Laboratory, New Haven, CT). Residue preferences at each position were originally assessed according to previously outlined methods (50, 51). Only signals that demonstrated a >50% increase in the absolute amount (picomoles) compared with the previous (or pre-previous) cycle were considered to indicate significant residues. Depending on the magnitude of signal increase seen for a given residue, these residues were classified as strong (>100%) or weak (>50%) residues (50, 51). A second pool of peptides that eluted as a predominant HPLC peak between 35 and 37 min was also separately collected and subjected to Edman degradation. An average relative frequency table was then generated by combining and converting the raw data from the two Edman degradation experiments (15–55 pool and 35–37 pool) according to the method of Kubo et al. (Ref. 52; data not shown). This average relative frequency table allowed for standardized values to be generated for individual runs and combined for comparison. No assignment of preferred amino acids was made for position 1 (P1), since this cycle tends to contain high levels of background signal, making proper assignments difficult. Cysteine was not modified during sequencing and therefore could not be detected.

Abbreviations used in this paper: Mamu, *Macaca mulatta*; B LCL, B lymphoblastoid cell line; TFA, trifluoroacetic acid.

Live cell binding assays

The live cell binding assays were performed as previously described (53). Briefly, Mamu-A*01-transfected 721.221 cells (10^6 cells/ml) were preincubated overnight in 5% FCS with 3 μ g/ml human β_2 -m (Scripps Clinic and Research Foundation, La Jolla, CA) at 26°C. Cells were washed twice in RPMI 1640 and resuspended to a concentration of 10^7 cells/ml. Cells (2×10^5 cells; 200 μ l/data point) were then incubated in the presence of 10^5 cpm (10 μ l) of a radiolabeled peptide, β_2 -m (3 μ g/ml), and where tested, 20 μ l of various concentrations of unlabeled inhibitor peptide at 20°C for 4 h. Peptides were HPLC purified and radiolabeled with 251 I according to the chloramine-T method (54). Following incubation, free and cell-bound peptides were separated by washing three times with serum-free medium and then passed through a FCS gradient. Pelleted fractions were counted on a gamma scintillation counter. In the case of competitive assays, the concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide was also calculated (IC_{50}) (55). JA2-K^b cells were used as a negative control cell line. These cells are stable transfectants of the human T cell leukemia line, Jurkat, expressing an HLA-A*0201/K^b fusion protein ($\alpha 1$ and $\alpha 2$ domains of HLA-A*0201 and $\alpha 3$ domain of H-2 K^b) (56).

Animals

Rhesus macaque rh95024 was identified as Mamu-A*01 positive by PCR-SSP and direct sequencing as previously described (57). Briefly, for allele-specific PCR, genomic DNA was isolated from peripheral blood using a QIAamp Blood Kit (Qiagen, Chatsworth, CA). DNA (50–150 ng) was then amplified in a single PCR reaction using two sets of Mamu-A*01-specific primers, Mamu-A*01F and Mamu-A*01R. rh95024 was then infected i.v. with 40 tissue culture infectious doses (TCID₅₀) of amplified SIVmac isolate 251. This animal was maintained in accordance with the National Institutes of Health Guide to the Care and Use of Laboratory Animals and under the approval of the University of Wisconsin Research Animal Resource Center review committee. Rhesus macaque rh118.87 was vaccinated with the live attenuated SIV strain SIVmac239 Δ nef (58) and housed at the New England Regional Primate Center (Southborough, MA).

Generation of B LCL lines

B LCL lines from rh95024 were generated by transformation of 1×10^6 freshly Ficoll/diatrizoate gradient purified PBL with equal volumes of S594 supernatant and R10 medium consisting of RPMI 1640 supplemented with penicillin (50 U/ml), streptomycin (50 μ g/ml), L-glutamine (2 mM), and 10% FBS (Biocell, Carson, CA). S594 supernatant was derived from a cell line productively infected with baboon *Herpes virus papio* (59).

Generation of cultured bulk CTL effector cells

PBL were isolated from whole blood using Ficoll/diatrizoate gradient centrifugation and then washed twice in R10 medium. CTL cultures were initiated by coculture of PBL with autologous B LCL stimulators expressing the SIV gag protein as previously described (60, 61). Stimulators were prepared by infecting 1×10^7 autologous B LCLs with a recombinant vaccinia virus construct expressing the gag gene of SIVmac 251 (vAbT252, a gift from D. Panicali and G. Mazzara, Thelon Biologies, Cambridge, MA). Four plaque-forming units per cell of recombinant vaccinia virus were used to infect B LCLs for 2 h in serum-free RPMI medium. Cells were then incubated in 10 ml of R10 medium, and the infection was allowed to continue for 14 h, at which time viable infected cells were collected by Ficoll/diatrizoate gradient centrifugation. Cells were then washed in HBSS (Life Technologies) and resuspended in 5 ml of 1.5% paraformaldehyde for 30 min at room temperature. Stimulators were spun down, resuspended in 5 ml of 0.2 M glycine in PBS, and incubated at room temperature for 15 min. Centrifuged cells were then washed once in 5 ml of FBS, and 5×10^6 stimulators were cocultured with 5×10^6 PBL in a 24-well plate (Corning, Corning, NY). Remaining stimulators were resuspended in FBS to a final cell concentration of 1×10^7 /ml and stored at 4°C until needed.

On day 3, 1 ml of the medium was replaced with R10 medium containing 20 U of rIL-2/ml (provided by M. Gately, Hoffmann-La Roche, Nutley, NJ). Medium was changed every other day with rIL-2 medium until day 7 when viable cells were purified on a Ficoll/diatrizoate gradient and resuspended in 2 ml of R10. Additional stimulators (5×10^6) were then added to the CTL cultures and incubated for another 3 days, after which half the medium was again replaced with rIL-2 medium every 2 days. On day 14 the CTL activity of the cultures was assessed in a standard 51 Cr release assay.

Isolation of CTL clones specific for the Mamu-A*01-restricted gag epitope

PBMC were obtained from rhesus macaque rh118.87. This animal, which had been vaccinated with the live attenuated SIV strain SIVmac239 Δ nef, had previously been shown to have strong CTL activity against the SIV gag protein (58). PBMC were stimulated initially with an autologous B LCL infected overnight with a recombinant vaccinia virus vector expressing SIV gag, pol, and env and then inactivated using UV light and psoralen. At 10 to 14 days, stimulated PBMC were depleted of CD4⁺ T cells using immunomagnetic beads as previously described (58). CD8⁺ T cells were then cloned by culturing them at 10, 3, and 1 cells/well in 96-well bottom plates in 200 μ l of RPMI supplemented with 5 μ g/ml Con A (Sigma), 20% FBS, 10 mM HEPES, 2 mM L-glutamine, 50 IU/ml penicillin, 10 μ g/ml streptomycin, and 100 U/ml recombinant human IL-2 (provided by M. Gately, Hoffmann-La Roche) in the presence of 1×10^5 irradiated (3,000 rad) human PBMC and 1×10^4 irradiated (10,000 rad) autologous B LCL as feeder cells. After 2 wk, wells exhibiting growth were restimulated with Con A, irradiated human PBMC, autologous B LCL, and IL-2 using a modification of techniques previously employed for the propagation of human HIV-specific CTL clones (17).

Peptides

Peptides were obtained as lyophilized products from Biosynthesis (Lexington, TX) or Chiron Mimotopes (San Diego, CA) or were synthesized Cytel using standard t-Boc or F-moc solid phase synthesis methods. Peptides synthesized at Cytel were reverse phase HPLC purified to >95% homogeneity. For CTL assays, lyophilized aliquots were resuspended in HBSS with 10% DMSO (Sigma) to a final concentration of 1 mg/ml. For live cell binding assays, peptides were resuspended at 20 mg/ml in 10% DMSO, then diluted with PBS.

Cytotoxicity assay

Testing of bulk CTL cultures required 5×10^5 autologous B LCL to be incubated for 1 h with 80 μ Ci of Na 51 CrO $_4$ (New England Nuclear Life Sciences Products) in a 200- μ l volume of R10 medium at 37°C in a 5% CO $_2$ humidified incubator. Target cells were then washed five times and resuspended to a concentration of 5×10^4 /ml in R10 medium. One hundred microliters of the target cell suspension was then plated into individual wells of a 96-well U-bottom microtiter plate. Peptide titrations were conducted by incubating the 51 Cr-labeled target cells with the indicated concentration of peptide for 1 h in individual wells of the 96-well plate before the addition of effector cells. Tenfold dilutions of peptides were made in HBSS with 10% DMSO. Effector CTLs were then added to replicate wells of target cells and allowed to coculture for 5 h before supernatants were harvested with a harvesting press (Skatron, Sterling, VA). 51 Cr release was measured on a Searle gamma counter (model 113; Searle, Skokie, IL). Spontaneous 51 Cr release was measured for each target cell line using four wells of target cells that received 100 μ l of R10. For additional wells received 100 μ l of 5% Triton X-100 (Sigma) and was used to measure maximum 51 Cr release. The percent specific lysis was determined using the following equation: % specific lysis = [(effector release – spontaneous release)/(maximum release – spontaneous release) \times 100]. Spontaneous release was always <25% of maximal release. Data reported for bulk CTL cultures are based on single CTL assays tested various E:T cell ratios.

CTL clones were tested for SIV-specific CTL activity using autologous B LCL or Mamu-A*01-transfected C1R cells (62), infected either with recombinant vaccinia virus vector expressing SIV gag and protease gene or with the unmodified vaccinia virus NYCBH as a control. Clones exhibiting SIV gag-specific activity were then screened for their ability to recognize autologous B LCL sensitized with the SIV gag peptide TPYTQML incubated with the B LCL during 51 Cr labeling at 100 μ g/ml. Peptide titrations were conducted by incubating the 51 Cr-labeled target cells with the indicated concentration of peptide for 45 min in individual wells of a 96-well plate before the addition of effector cells as previously described (63). For each CTL clone the pattern of recognition of these different peptides was confirmed in three or more CTL assays.

Results

Peptides bound by Mamu-A*01 possess a strong proline anchor at the third position

To assist in the correct identification of additional Mamu-A*01-restricted CTL epitopes, we were interested in determining anchor residues of peptides bound by Mamu-A*01. To accomplish

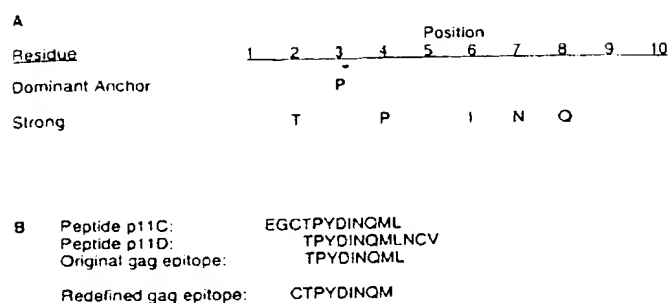


FIGURE 1. A, The peptide binding motif of Mamu-A*01. This motif is based on the results obtained from Edman degradation analysis of two sets of pooled peptides eluted from Mamu-A*01. The results indicate that a dominant proline anchor residue exists at position 3. Amino acids preferred at other positions are also shown. The signal for proline in cycle 3 was much more significant than signals for any other amino acid in this or any other cycle and was therefore determined to represent the only anchor residue for Mamu-A*01. B, Redefinition of the Mamu-A*01-restricted SIV gag CTL epitope. The SIV gag CTL epitope was originally mapped to a 9 mer peptide based on CTL responses to two overlapping 12-mers, p11C and p11D (16). Determination of the peptide binding motif of Mamu-A*01 indicated that the proline residue occupied P3 rather than P2, suggesting, therefore, that a cysteine residue occupies P1 of the SIV gag CTL epitope.

this, Mamu-A*01 was transfected into the human MHC class I-deficient B LCL line 721.221. Peptides bound by this molecule were then eluted and sequenced by Edman degradation. Sequence analysis of the first set of pooled peptides, which eluted between fractions 15 and 55, revealed a striking enrichment of the signal for proline at position 3 (P3), which was greater than any other signal identified (data not shown). From the combined data of this and an Edman degradation run of a second pool of peptides, other weaker enrichments, possibly reflecting secondary anchor residues, were confirmed for threonine at P2, for proline at P4, for isoleucine at P6, for asparagine at P7, and for glutamine at P8 (Fig. 1A). Increases in signal for other amino acids were also observed at other positions; however, these signals were not consistently elevated in the two Edman degradation experiments and were not considered significant. Therefore, the proline residue that occupies P3 of the SIV gag CTL epitope appears to represent the anchor residue critical for the binding of the SIV gag epitope to Mamu-A*01. These findings suggest that the naturally processed peptide may span residues 181 to 190 (or possibly 181–189). This differs from the previously described SIV gag Mamu-A*01-restricted CTL epitope (TPYDINQML_{182–190}) in that the redefined epitope possesses an additional NH₂-terminal residue (C₁₈₁; Fig. 1B).

*The P1 cysteine residue of the Mamu-A*01-restricted SIV gag epitope is required for high affinity binding to Mamu-A*01*

Since the peptide binding motif data suggested that the Mamu-A*01-restricted SIV gag CTL epitope possessed a proline residue at P3 rather than at P2, as previously described, we were interested in using binding assays to test the validity of these findings. We examined the binding abilities of a panel of SIV gag_{181–191}-derived peptides in a direct live cell binding assay specific for Mamu-A*01. Since a cysteine residue can interfere with proper radiolabeling of these peptides, all the analogues tested contain an alanine to cysteine substitution at P1. The 10 mer peptide (ATPYDINQML) bound to 721.221 cells transfected with Mamu-A*01, but not to untransfected 721.221 cells or 721.221/A2-K^b cells. Furthermore, when an excess of unlabeled homologous peptide was added to inhibit binding, it was determined that binding of the labeled ATPYDINQML peptide was of high affinity (IC₅₀ = 3.1

Table 1. Mamu-A*01 binding capacity of a panel of SIV gag_{181–191} truncations

| Peptide | Sequence | Length (bp) | IC ₅₀ (nM) |
|-----------------|-------------|-------------|-----------------------|
| SIV gag 181–191 | CTPYDINQMLN | 11 | 703 ^a |
| SIV gag 181–190 | CTPYDINQML | 10 | 5.9 |
| SIV gag 181–189 | CTPYDINQM | 9 | 4.3 |
| SIV gag 181–188 | CTPYDINQ | 8 | 19936 |
| SIV gag 182–190 | TPYDINQML | 9 | 112 |
| SIV gag 182–189 | TPYDINQM | 8 | 528 |
| SIV gag 183–190 | PYDINQML | 8 | 10886 |
| SIV gag 183–189 | PYDINQM | 7 | >30000 |

^a IC₅₀ values represent the concentration of test peptide required to outcompete 50% of the radiolabeled peptide.

nM; data not shown). Other unrelated peptides also inhibited binding of the radiolabeled peptide to various degrees, but with binding affinities ranging from about 100 nM for the HBV_{18–27} analogue (FLPSDYFPSV), which also carries a proline in position 3, to undetectable levels in the case of the rat 60s L28 peptide (FRYN GLIHR; data not shown).

We then analyzed the abilities of several SIV gag_{181–191}-derived peptides to bind to Mamu-A*01 as measured by inhibition assays. Since MHC class I molecules are able to bind peptides of variable length, it was necessary to define the length of this epitope using additional truncated peptides. It was first determined that the natural peptide (CTPYDINQML) bound Mamu-A*01 as well as the C > A analogue, demonstrating an IC₅₀ value of 5.9 nM (Table I). Examination of COOH-terminal truncated SIV gag peptides revealed optimal binding with either 9 mer (CTPYDINQM) or 10 mer (CTPYDINQML) peptides. This may indicate that both M₁₈₉ or L₁₉₀ are capable of being bound by the F pocket of Mamu-A*01. Elongation of the peptide to include an additional COOH-terminal residue (N₁₉₁), however, dramatically increased the IC₅₀ value, thereby decreasing the binding affinity. Likewise, further truncation of the 9 mer peptide by removal of the COOH-terminal M₁₈₉ residue dramatically reduced binding to Mamu-A*01.

Next, examination of NH₂-terminal truncation of the SIV gag peptides revealed that loss of the NH₂-terminal cysteine (C₁₈₁) decreased the binding affinity of the peptides ending with the leucine (L₁₉₀) or methionine (M₁₈₉) residues by 19- and 123-fold, respectively. These data demonstrate an important role for the cysteine residue in binding of this epitope to Mamu-A*01 and support the assignment of the proline residue to position 3 of this epitope, rather than to P2 as in the originally proposed epitope. Additional experiments demonstrated that further truncation of the NH₂-terminal threonine (T₁₈₂) almost completely abolished binding. In conclusion, these results suggest that the SIV gag peptide associated with optimal Mamu-A*01 binding capacity is either a 9 or 10 mer peptide beginning with the C₁₈₁ residue.

Binding of single substitution analogues reveals that the P3 proline represents the dominant anchor residue

To verify the peptide binding motif determined for Mamu-A*01, single substitution analogues of the C > A peptide analogue (ATPYDINQML) were tested in live cell binding assays. Initially, lysine residues were substituted at all positions to determine whether substitution with this larger, charged residue affected binding. As illustrated in Table II, analogues with lysine substitutions at P2, P3, P8, and P10 had significantly reduced binding capacity. Additional analogues tested at P2 revealed that a variety of amino acid substitutions at this position were not well accommodated and resulted in reduced binding. However, a few substitutions, including alanine, proline, and valine were accommodated. This was not

Table II. Mamu-A*01 binding capacity of a panel of single substitution analogs of the Mamu-A*01 binder 1279.06

| Peptide | Sequence | | | | | | | | | | Binding Capacity (IC ₅₀ , nM) |
|---------|----------|---|---|---|---|---|---|---|---|----|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| 1279.06 | A | T | P | Y | D | I | N | Q | M | L | 3.3 |
| F130.01 | K | | | | | | | | | | 9.0 |
| 33.0016 | | A | | | | | | | | | 5.6 |
| 33.0020 | | P | | | | | | | | | 7.4 |
| 33.0017 | | V | | | | | | | | | 8.0 |
| 33.0019 | | Q | | | | | | | | | 62 ^a |
| 33.0018 | | F | | | | | | | | | 174 |
| F130.02 | | K | | | | | | | | | 1243 |
| F130.05 | | | V | | | | | | | | 20 |
| F130.03 | | | A | | | | | | | | 26 |
| F130.06 | | | F | | | | | | | | 35 |
| F130.08 | | | Q | | | | | | | | 52 |
| F130.04 | | | T | | | | | | | | 57 |
| F130.07 | | | K | | | | | | | | 78 |
| F130.09 | | | | K | | | | | | | 5.3 |
| F130.10 | | | | | K | | | | | | 4.6 |
| F130.11 | | | | | | K | | | | | 8.2 |
| F130.12 | | | | | | | K | | | | 5.7 |
| 33.0021 | | | | | | | | N | | | 4.4 |
| 33.0023 | | | | | | | | A | | | 4.7 |
| 33.0024 | | | | | | | | F | | | 6.1 |
| 33.0022 | | | | | | | | I | | | 11 |
| F130.13 | | | | | | | | K | | | 14 |
| F130.14 | | | | | | | | | K | | 5.0 |
| F130.15 | | | | | | | | | I | | 2.8 |
| F130.16 | | | | | | | | | M | | 4.6 |
| F130.17 | | | | | | | | | F | | 5.3 |
| F130.20 | | | | | | | | | A | | 6.0 |
| F130.18 | | | | | | | | | T | | 8.6 |
| F130.19 | | | | | | | | | Q | | 14 |
| F130.21 | | | | | | | | | K | | 48 |

^a Bold type indicates three times or greater decrease in binding capacity.

unexpected, since valine was also revealed as a possible weak P2 anchor in the peptide binding motif of Mamu-A*01 (data not shown), and alanine often accompanies motifs with threonine/valine anchors. However, similar analogues tested at P3 revealed that no residue could replace proline, underlying the role of proline as a crucial anchor residue. P8 and P10 lysine substitution analogues were the only other analogues that revealed decreased binding capacity. However, unlike the P2 and P3 analogues, only those P8 and P10 analogues containing lysine (as well as glutamine for P10) had reduced binding. Many other P8 and P10 analogues tested did not significantly alter IC₅₀ values, suggesting that, while P8 and P10 residues are important for binding to Mamu-A*01, it is unlikely that strong anchor residues exist at these positions.

The SIV gag₁₈₁₋₁₉₉ peptide sensitizes target cells for lysis even in nanogram amounts

It is often difficult to appreciate even dramatic differences in peptide recognition by CTLs among sets of peptides if high peptide concentrations are used to pulse target cells. At these high concentrations, even suboptimal peptides may be capable of inducing appreciable levels of specific lysis in the CTL assays. Therefore, in the next series of experiments we used bulk CTL lines derived from a Mamu-A*01-positive SIV-infected rhesus macaque to determine the optimal CTL epitope through testing of various dilutions of peptides for their ability to induce lysis of target cells.

Initially we tested the ability of the 9-mer (CTPYDINQM) and 10-mer (CTPYDINQML) peptides, the optimal Mamu-A*01 binders, for this capacity to sensitize Mamu-A*01-transfected 721.221 target cells for lysis. As illustrated in Figure 2, A and B, only minor

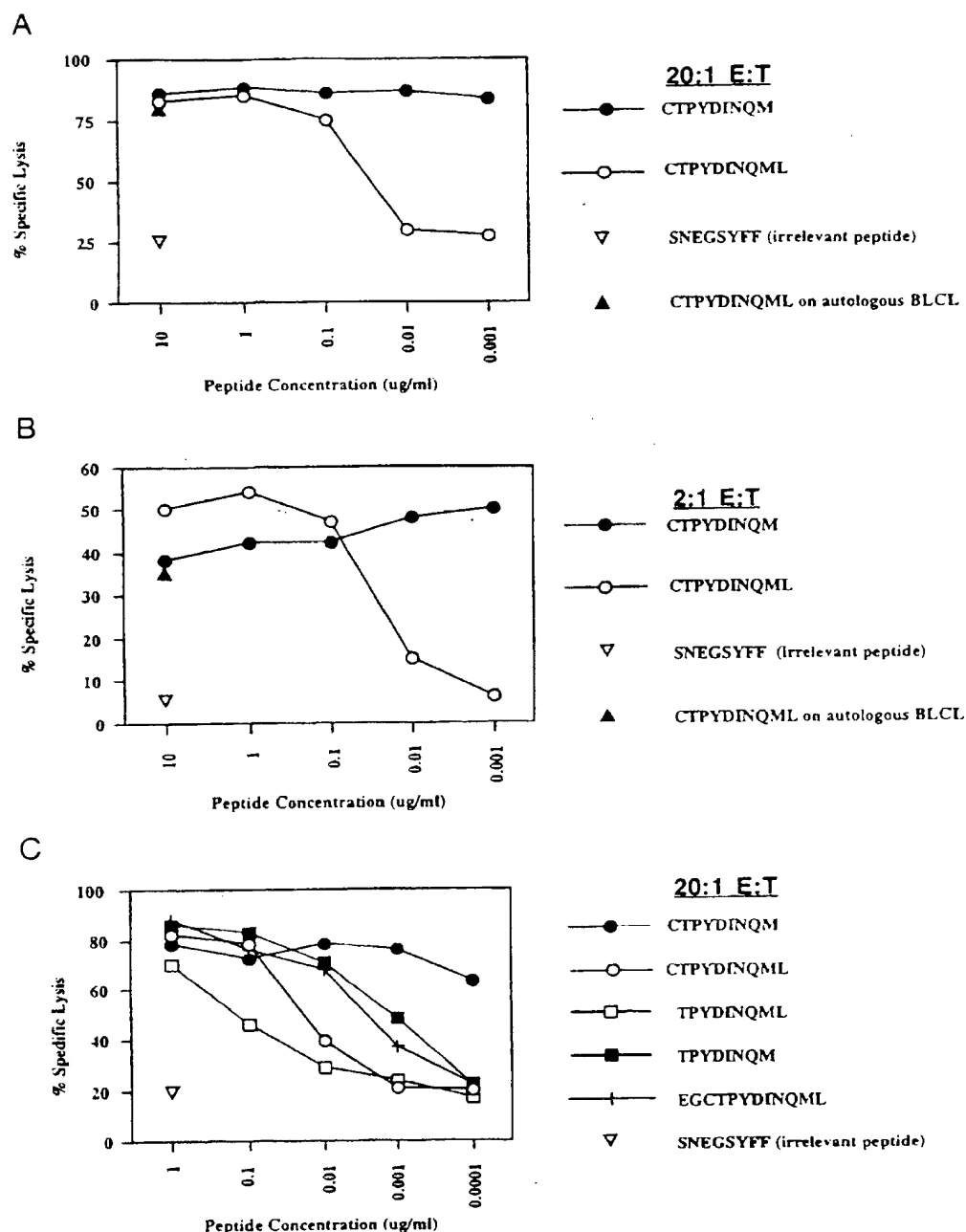
differences in the percent specific lysis were observed at high peptide concentrations. However, when nanogram amounts of the peptides were tested, the 9-mer was >100-fold more active than the 10-mer. Bulk CTL lines were also shown to recognize autologous B LCLs equally as well as Mamu-A*01-transfected 721.2 cells when pulsed with high concentrations of peptide. These peptides were then retested along with the 8-mer TPYDINQM, 9-mer TPYDINQML, and 12-mer EGCTPYDINQML peptides (Fig. 2C). The 9-mer CTPYDINQM yielded the highest levels of CTL activity at low peptide concentrations and was almost 1,000-fold more active than the 10-mer CTPYDINQML. Furthermore, the 9-mer CTPYDINQM peptide appeared to be recognized 100-10,000-fold better than the two other truncated peptides, TPYDINQM and TPYDINQML, which were missing the crucial cysteine residue at position 1. The p11C 12-mer (EGCTPYDINQML), originally used to map the Mamu-A*01-restricted SIV gag epitope, was also poorly recognized at low peptide concentration. These findings suggest that the SIV gag CTL epitope ends in methionine residue and identifies the SIV gag CTPYDINQM peptide as the optimal CTL epitope for recognition by bulk CTL lines.

Mamu-A*01-restricted SIV gag CTL clones differentially recognize the CTPYDINQM and CTPYDINQML peptides

We next examined recognition of a panel of SIV gag peptides by three SIV-specific CTL clones obtained from a Mamu-A*01-positive rhesus macaque that developed a CTL response dominated by the specificity for the 25-mer gag peptide 11, spanning residues 171 to 195 (16, 58). Initial screening of 106 gag-specific CTL clones from this animal with the previously reported 9-mer (TPYDINQML₁₈₂₋₁₉₀) revealed that 92 clones (87%) recognized this peptide. Two of these clones (no. 8 and 20) were randomly selected for further study as representative clones. An additional clone (no. 125), which did not recognize the previously described minimal epitope (TPYDINQML) but did recognize the 25-mer gag peptide 11 (data not shown), was also used for these studies. Recognition of various gag peptides over a range of peptide concentrations was examined using autologous B LCLs and Mamu-A*01-transfected C1R cells. Although each of the CTL clones presented a distinct pattern of recognition with this panel of peptides, for all clones the optimal epitope consisted of either the 9-mer CTPYDINQM or the 10-mer CTPYDINQML (Fig. 3, A and B). In general, the 9-mer TPYDINQML was recognized by these clones 10- to 10,000-fold less efficiently than the CTPYDINQM or CTPYDINQML peptides. Clone 125 did not recognize the 9-mer peptide TPYDINQML at any concentration tested, except when tested on Mamu-A*01-transfected C1R cells at very high peptide concentrations (Fig. 3B). Interestingly, clone 8 did not recognize the 9-mer CTPYDINQM peptide at all, while it recognized the 10-mer CTPYDINQML with a sensitizing dose of peptide required for 50% maximal lysis of 0.001 µg/ml. This finding is in agreement with the peptide binding data examining lysine analogues (Table I) which suggested that the P10 leucine of the 10-mer (CTPYDINQML) could also be bound by the F pocket of Mamu-A*01. However, both clones 125 and 20 recognized the CTPYDINQM peptide as the optimal epitope. Thus, while there was variation in the optimal epitope recognized by each of these clones, the consensus optimal epitope consisted of either the 9-mer CTPYDINQM or the 10-mer CTPYDINQML with the proline residue at position 3.

Discussion

We have defined the first peptide binding motif of a nonhuman primate MHC class I molecule, Mamu-A*01. Knowledge of this molecule's peptide binding motif has allowed us to redefine the



minimal Mamu-A*01-restricted SIV gag CTL epitope. Although we observed some variation among different CTL clones, the consensus optimal epitope recognized by bulk CTL cultures and CTL clones has now been mapped to residues 181 to 189 (CTPYDINQM) of the gag protein and differs from the originally defined epitope believed to lie between residues 182 and 190 (TPYDINQML) (16, 45, 64). The corrected optimal epitope was determined by peptide elution, live cell binding assays, and dilutions of peptides tested in CTL assays. The accurate identification of this optimal CTL epitope will facilitate future CTL studies in SIV-infected rhesus macaques.

CTLs from Mamu-A*01-positive rhesus macaques were previously shown to recognize two overlapping 12-mer peptides, p11C and p11D (16). These two 12-mers overlapped by nine residues, and it was therefore concluded that the minimal CTL epitope was TPYDINQML₁₈₂₋₁₉₀ (16, 45, 64). However, we have demonstrated

that gag-specific bulk CTL cultures recognized Mamu-A*01-positive target cells pulsed with the SIV gag CTPYDINQM peptide even at nanogram amounts, whereas much greater concentrations of the TPYDINQML, or the longer CTPYDINQML, peptides were required to obtain similar levels of recognition. Studies with cloned CTL confirmed this hierarchy of CTL recognition, with the CTPYDINQM peptide being recognized by the majority of the CTL clones at low peptide concentrations.

It is interesting that one CTL clone (no. 8) preferentially recognized the longer CTPYDINQML peptide and, in fact, failed to recognize the CTPYDINQM peptide even at concentrations up to 10 μ g/ml. This finding combined with our peptide binding data that demonstrated little difference between binding of this 10-mer and the minimal epitope CTPYDINQM, as well as data demonstrating a role for the P10 leucine in binding of the 10-mer, suggest that Mamu-A*01 may be capable of binding and presenting both

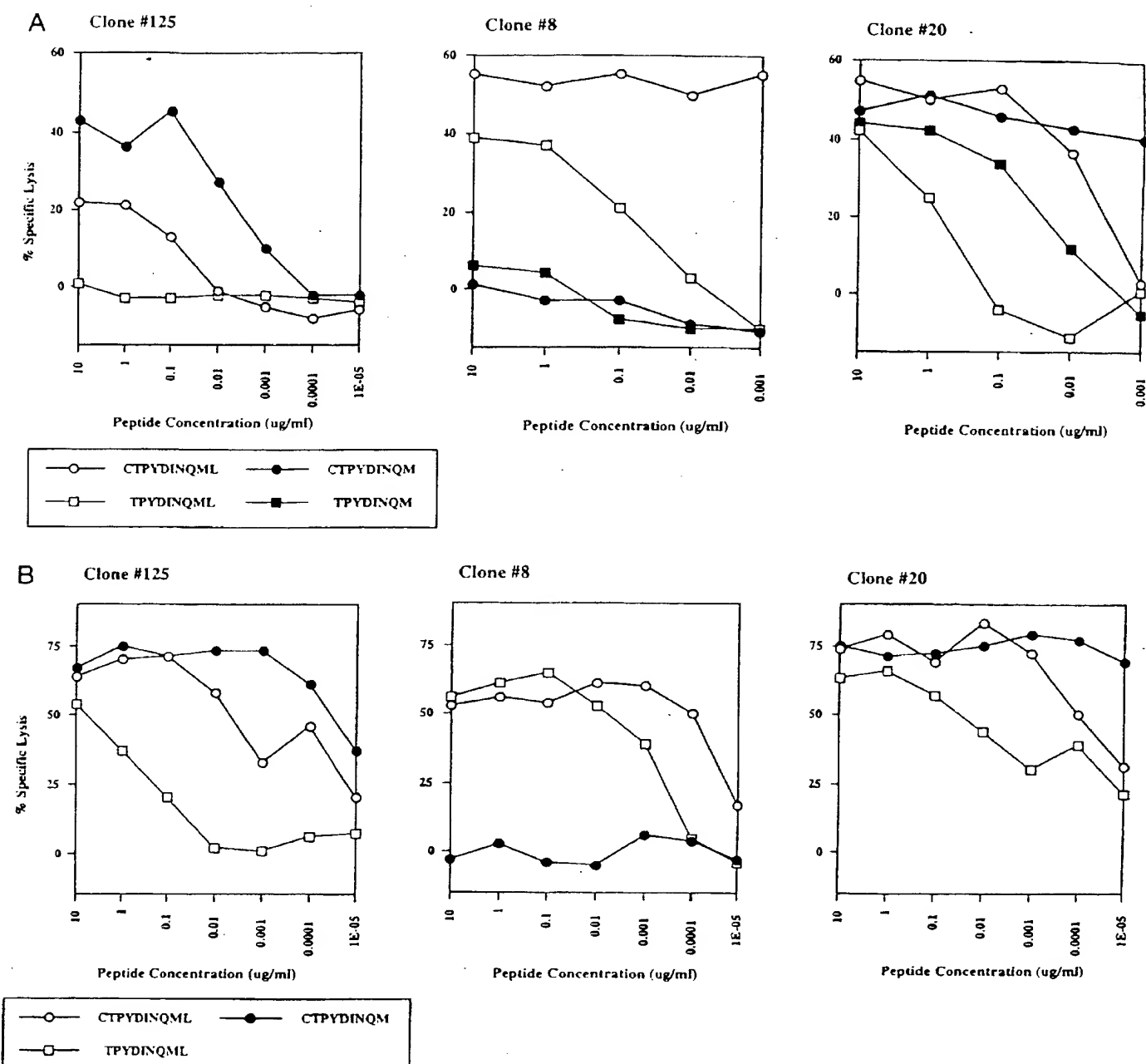


FIGURE 3. Two of three CTL clones preferentially recognize the SIV gag 9 mer CTPYDINQM. Mamu-A*01-restricted gag-specific CTL clones were tested at an E:T cell ratio of 5:1 against four different SIV gag peptides using various peptide concentrations. **A**, Target cells were peptide-pulsed B LCLs. **B**, Target cells were peptide-pulsed Mamu-A*01-transfected C1R cells.

of these peptides. Therefore, SIV-infected Mamu-A*01-positive rhesus macaques may be capable of mounting significant CTL responses against both these epitopes. The preferential reactivity of bulk CTL cultures toward the shorter epitope, however, may be a reflection of higher precursor frequencies of circulating CTLs specific for the shorter CTPYDINQM peptide. This may be due to subtle differences in the MHC class I Ag processing pathway that might favor production of the shorter epitope from the SIV gag protein.

Identification of this first rhesus macaque MHC class I peptide motif (Mamu-A*01), proved to be interesting in that peptides bound by Mamu-A*01 possess a single dominant anchor residue at

position 3 (P3). The majority of human MHC class I molecules, however, bind peptides with anchor residues at positions 2 (P2) and 9 (P9) that interact with the B and F pockets of an MHC class I molecule's peptide binding groove (65). The presence of a P3 anchor for Mamu-A*01 suggests that the D pocket, which binds P3 residues, is responsible for tight binding of peptides to this molecule (66–68). Interestingly, the D pocket is rather shallow for the majority of human MHC class I molecules and is believed to provide less binding energy than the B and F pockets for the peptide-MHC complex (66–68). Position 3, however, does appear to be an important secondary anchor site for a number of HLA alleles (69–71). Furthermore, despite the shallowness of the D pocket, a

few mouse (H-2D^d) and human (HLA-A*01, -B*08) MHC class I molecules possess a dominant anchor motif at P3. Analysis of the B pockets of Mamu-A*01 and these other mouse and human MHC class I molecules, which do not possess P2 anchor motifs, reveals that some of the residues forming their B pockets are unique to these molecules and may interfere with the ability of their B pockets to tightly bind P2 residues (data not shown). As such, in these molecules the D pocket may supplant the role of the B pocket by tightly binding P3 residues. Determination of the peptide binding motifs of additional rhesus macaque MHC class I molecules will be necessary to determine whether the absence of P2 anchor motifs is a general characteristic of these molecules.

The absence of a strong COOH-terminal anchor, usually at P9, in the defined peptide binding motif of Mamu-A*01 is surprising. However, live cell binding assays indicated that loss of the COOH-terminal methionine of the shorter peptide (CTPYDINQM) dramatically reduced the ability of this peptide to bind Mamu-A*01. This strongly supports a role for COOH-terminal residues of peptides in binding to Mamu-A*01. Indeed, preliminary analysis of the F pockets of various rhesus MHC class I molecules, including Mamu-A*01, suggests that residues forming the F pockets of these molecules are very similar to those of their human counterparts and, therefore, would be expected to possess strong P9 anchors.

It has been observed that human and rhesus macaque MHC class I molecules bind CTL epitopes from similar regions of HIV and SIV, respectively. For example, the rhesus Mamu-A*01-restricted SIV gag epitope was originally believed to be identical with the HLA-B*53-restricted epitope from the HIV-2 gag protein (TPYDINQML) (15). Similarly, other SIV epitopes bound by Mamu-A*08, -B*01 and -A*02 (18, 19, 72) overlap significantly with previously described HIV epitopes restricted by the human MHC class I molecule HLA-A*02. However, closer examination of the residues forming the B (and D) pockets these MHC class I molecules reveals that these human and rhesus molecules do not possess similar pockets and would not be expected to bind the same minimal epitopes. Therefore, basing assumptions regarding SIV CTL epitopes on closely related HIV epitopes may be misleading, and defining the peptide binding motifs of these rhesus MHC class I molecules will be necessary for the accurate identification of their minimal SIV CTL epitopes.

Peptide immunizations of Mamu-A*01-positive rhesus macaques have been attempted previously on several occasions (44, 73, 74). However, although these peptide immunizations generated a CTL response, they were never able to protect rhesus macaques from subsequent infection with pathogenic virus. Unfortunately, in all these experiments, a 12-amino acid peptide (p11C) was used to immunize these rhesus macaques. We have now determined that the optimal peptide bound by Mamu-A*01 is only 9 to 10 amino acids in length. Since the immunogenicity of a peptide is crucially dependent on its affinity for MHC class I molecules (75), it is possible that in these previous experiments the CTL responses generated were not optimal. Thus, immunization with smaller peptides that bind to MHC class I molecules with higher affinity may improve CTL responses compared with immunization with longer peptides that have a much lower affinity for the MHC class I molecule.

Mamu-A*01-positive rhesus macaques make an immunodominant response to the SIV gag protein. In previous studies, each of five SIVmac-infected rhesus macaques developed CTLs against this protein of the virus, and in each case Mamu-A*01 was the restricting MHC class I molecule (16). Furthermore, only a single peptide (p11, a 25 mer) was recognized significantly. These findings, combined with the frequency of Mamu-A*01, which is >22% in rhesus macaques originating from India (16, 57, 76),

make this epitope an obvious choice for vaccines designed to induce CTLs against this region of the virus. Indeed, this region of the SIV gag protein appears to be well conserved in nine different SIV isolates derived from rhesus macaques, stump-tailed macaques, and sooty mangabeys, with only a single position 9 (M→L) variant existing in a stump-tailed macaque isolate (data not shown). In this study we have redefined a previously reported CTL epitope from the SIV gag protein. This epitope can now be used to develop epitope-based vaccines, to detect viral escape mutants, or to form soluble peptide-MHC dimers or tetramers (77, 78) for the identification and characterization of peptide-specific CTL.

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